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FIRST ANNUAL REPORT

February 1, 1992 - January 31, 1993

"Evaluation of Dried Storage of Platelets for Transfusion:  
Physiologic Integrity and Hemostatic Functionality"

Grant No. N00014-92-J-1244  
From the Office of Naval Research:  
Navy Medical Research and Development Command  
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Performance Sites and Investigators:

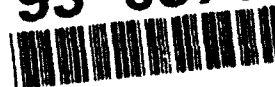
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Respectfully submitted, April 21, 1993

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#### Administrative Review:

The first year's administrative activity involved preparation of subcontract agreements between the performance sites, acquisition of new equipment and personnel, and filing of a U.S. Patent application on lyophilized platelets. The subcontract spending authority was established by the end of the first quarter; work done in the first few months of the project was covered by funds held over from the prior grant (No. N00014-89-J-1712) or by backdating expenditures to the start date of the new grant (Feb. 1, 1992). The new equipment acquired included a Virtus 600 Lyophilization unit for preparation of lyophilized platelets at ECU. This purchase provided a second production site to facilitate further development of stabilization protocols. The application for a patent was filed in the U.S. Patent and Trademark Office on May 29, 1992 (application serial number 07/891,277). The first review by the Patent Office was received without acceptance of claims; a continuation-in-part is in preparation at this time. The University of North Carolina has drafted a license agreement with the Armour Pharmaceutical Division of Rhone-Poulenc Rorer for exercise of options after a patent is obtained on preparation of a lyophilized transfusion product.

#### Scientific Review:

The experimentation at UNC-Chapel Hill is summarized in the attachment from Dr. Read. The intramural collaboration at ECU to study metabolic activity of lyophilized platelets was canceled after six months due to departure of the investigator. This study will now be carried out by colleagues at the American Red Cross Research Laboratory in Norfolk, VA. Samples of various platelet preparations have been sent and analysis is underway. The major objectives achieved at ECU in the first year were initiation of long-term storage studies of the standard lyophilized platelet preparation, set-up of Baumgartner chamber methodology for assessment of platelet adhesiveness, and further development of permanganate-based stabilization protocols before platelet lyophilization.

A long-term study of the stability of surface receptors on lyophilized platelets stored under various conditions (desiccated, at room temperature or 4°C, or frozen at -70°C) was initiated in June, 1992, with a single large batch of paraformaldehyde-treated dried human platelets. At one month intervals, sample vials from each storage environment were reconstituted and assayed by flow cytometry with monoclonal antibody probes to the major surface glycoproteins (GPIb, GPIbIX complex, GPIIbIIIa, and GPIV). The only significant finding thus far (6 months) is a decrease in binding of MoAb SZ-1 to the dried platelets stored at room temperature. This antibody binds to the GPIbIX complex but not to GPIb or GPIX separately; the loss of this epitope on stored platelets may indicate a denaturation of the complex. Further studies will be necessary to determine the effect of this change on functionality of the platelets stored at R.T.; such as adhesion or VWF binding. The dried platelets stored at 4°C or -70°C have not shown any evidence of change.

The continuing development of alternative protocols for preparing lyophilized platelets necessitated the purchase and installation of a Virtus 600 Lyophilizer for use at ECU. As part of the analytical comparison of batches, we have also begun performing Baumgartner chamber perfusion experiments to test the adhesive qualities of human lyophilized platelets on canine arterial vessel strips. In the latter part of this first year of this project, we ran fifteen Baumgartner studies on platelets stabilized with various protocols of paraformaldehyde or permanganate in different buffers and cryoprotectants. Adhesion of platelets was assessed by morphometric analysis of microphotographs taken of exposed vessel strips treated with the P-2 MoAb to reveal platelets by epifluorescence. Fresh platelets covered 53-76% of the exposed vessel area, while different preparations of lyophilized platelets have ranged from 23-80% coverage. Besides these differences in adhesion, we have found that the effect of the experiment on platelets remaining unattached to the vessel can show differences among batches. Non-adherent platelets in fresh whole blood show signs of activation (GMP-140 surface expression) after being circulated in the Baumgartner chamber. Several of the lyophilized platelet preparations show evidence of activation also; the percentage of platelets positive for GMP-140 expression increased 2-3 fold during the perfusion study. This is a very significant finding in that it suggests that these platelets are capable of responding appropriately to physiologic stimuli.

The practical aspects of freeze-drying platelets in unit doses were addressed during this first year. We have continued to refine stabilization protocols employing permanganate instead of paraformaldehyde for the advantage of shortening the processing time. At present, the centrifugation and resuspension steps are carried out in an open system, and our first attempts at completing the stabilization steps within the closed system of a blood collection set revealed problems in the number of entries into the bag to add fluids or remove wash supernates. We are currently devising ways to reduce the number of transfers and washes to make sterile processing more feasible. If necessary, sterile preparations can be produced as needed under a laminar flow hood, but this may not be practical at blood centers.

In summary, we are progressing towards a product suitable for clinical trials. Experiments are underway to test the efficacy of lyophilized platelets in thrombocytopenic animal models, and further analysis is being conducted in vitro to assess the effects of processing on the activatability and metabolism of current preparations. In the next year, more emphasis will be placed on in vivo circulatory lifespan determinations, sterile processing, and interactions of lyophilized platelets with the fibrinolytic system. We hope these efforts are deemed satisfactory. The depth of details on data and methods has been deliberately reduced in this report in response to the Patent Office's concerns of the discoverable nature of these documents. The investigators will be happy to supply further in-depth analysis if desired as long as confidentiality can be maintained.

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February 1, 1992 - January 31, 1993

Grant No. N00014-92-J-1244  
The Office of Naval Research  
Department of the Navy

P.I./ECU: A. P. Bode, Ph.D.

Contract: UNC/ECU  
Dehydration of Platelets and RBC; Long-Term Storage of  
Transfusion Products.

P.I./UNC: Marjorie S. Read, Ph.D., University of North  
Carolina- Chapel Hill, North Carolina.

Co. P.I. Robert L. Reddick, M.D., UNC-CH

Submitted April 19, 1993

Experiments conducted during 1992.

Major groups of studies conducted.

1. In Vivo studies: 5 normal dogs ( 6 experiments), 2 vWD dogs, 4 normal pigs and 3 vWD pigs (5 experiments).

2. In Vitro studies: surface antigens present on rehydrated platelets.

3. Clinical use of rehydrated platelets to correct bleeding in one vWD pig.

Results:

1. In vivo studies.

Two types of studies were conducted: a) Rehydrated platelets were infused into normal and bleeder animals to repeat and confirm results previously reported with the stenosis and injury model. Several experiments were needed to establish that rehydrated platelets bind only to thrombogenic subendothelium and areas where the vessel has been injured. In the normal animals results were confirmatory. Rehydrated platelets adhere to thrombogenic surfaces and do not adhere to normal, uninjured vessels. In both pigs and dogs, platelets were found in the clots formed from flowing blood at bleeding time sites and in thrombi formed within areas of induced vessel wall injury and disruption.

In bleeder animals, few platelets were seen adherent to injured areas unless vWF was added by transfusions of plasma or cryoprecipitate rich in vWF. These findings indicate

that rehydrated platelets are not more thrombogenic than fresh platelets and do not form abnormal thrombi in this model. Rehydrated platelets are dependent on von Willebrand factor and GPIb for adhesion.

b) Rehydrated platelets were infused into normal and bleeder animals to evaluate circulation. In these studies the platelets were labeled with fluorescent dye and not with a radiolabel. Small numbers of platelets were infused, from 10 to 30 % of the total original platelet count. There was generally a decrease in immediate post infusion platelet count with return to normal within 2 hours. Platelets were detected in dog PRP for 24 hrs after the infusion, but not in the pig. Dried platelets prepared from a vWD pig were transfused into both normal and bleeder pigs to determine if vWF compartmentalized in the platelets, absent in plasma and vessel wall, played a role in the removal of rehydrated pig platelets from circulation. Both normal and vWD rehydrated platelets failed to circulate in the pig. Rehydrated platelet transfusions accompanied by transfusion of cryoprecipitate showed good circulation of labeled rehydrated platelets. The greatest yield of circulating rehydrated platelets was in a vWD pig given rehydrated platelets followed by cryoprecipitate followed by a second rehydrated platelet infusion. Studies of platelets dried in the plasma are under way. Studies of dried platelets rehydrated in plasma, either autologous or heterologous

plasma, are also being done to evaluate recovery and ability such platelets to circulate.

## 2. In Vitro studies.

Rehydrated platelets are being studied with a bank of antibodies to mark surface antigens. The following antibodies are being used to look at proteins fixed to the platelet surface: antibodies against PDGF, vWF, Factor XIII, Factor VIII, Factor IX, GMP 140, Fibrinogen, Fibronectin, and Plasmin are included in the study. The presence of granule proteins on the rehydrated platelet surface will be used as an indicator of activation state of rehydrated platelets. PDGF, FXIII, and plasmin have been found to be prominently displayed on single rehydrated platelets. Rehydrated platelets agglutinated with botrocetin display more antigen indicating secretion and/or binding of plasma proteins during agglutination. However, since botrocetin is specific for vWF and vWF induced agglutination of fixed platelets involves only the platelet membrane and GP receptors, the increase presence of granule proteins may be the result of release by the stabilized platelet. We have mixed results (data not interpretable) to date on the effect of bound plasmin and fibrinogen.

The presence of bound PDGF stimulated additional investigation into the ability of rehydrated platelets to promote, enhance and speed wound repair. We have studied control wounds in 2 pigs. The histologic data indicate that dried platelets stimulate growth of fibroblast and speed

repair. Mesh bandages impregnated with dried platelets were more efficient than direct applications of "powdered" platelets. This may be due the fact that the mesh holds platelets in place as opposed to layering of the platelet powder. This unexpected finding may prove to be a cheap source of PDGF that can aid in repair for patients with large open wounds such as burn patients.

We are presently involved in studies of dried platelets and the formation of prothrombinase complex. Preliminary data indicates that lyophilized platelets perform as well as fresh platelets in these studies.

### 3. "Clinical" Studies.

We have used rehydrated platelets in bleeder pigs to aid in control of clinical bleeds. In one vWD pig with an uncontrolled bleed from the nose, rehydrated platelets in conjunction with cryoprecipate (cryo) stopped the bleeding. This pig was initially treated with plasma then cryo. The bleeding was unabated with either procedure. Rehydrated platelets alone likewise did not stop bleeding. Only when rehydrated platelets and cryo were given at the same time was bleeding stopped. This result is the same as that seen by Nichols et al in vWD pigs. In their studies, bleeding time was corrected only with fresh platelets and plasma or cryo. Plasma or cryo alone, in their studies, did not correct the porcine bleeding time.



We have ongoing studies using the Xylum Clot Signature Analyzer to evaluate the role of dried platelets in blood clotting. The data to date are variable but encouraging. Rehydrated platelets appear to have a similar bleeding time and clotting time to fresh platelets.

On the whole, studies with rehydrated platelets have been very successful. We have had two troublesome experiments in dogs, however. In two dogs, infusions of rehydrated platelets caused weakness, loss of platelets, and temperature rise. Dogs were treated with benadryl or epinephrine and both recovered. None of the other normal and vWD animals, pigs or dogs, have had any reaction to rehydrated platelets. The preparations for these two studies were made by two new student workers under the supervision of the lab technician. We are evaluating these data to determine if the method of preparation varied from the other preparations. One of the dogs still resides in the kennel and will be tested again for reaction to other preparations.